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PRINCIPAL INVESTIGATOR: Albert S. Baldwin, Ph.D.

CONTRACTING ORGANIZATION: University of North Carolina at Chapel Hill
Chapel Hill, North Carolina 27599-4100

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Albert S. Baldwin, Ph.D.

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13. ABSTRACT *(Maximum 200 words)*

Human breast cancer is characterized by the inappropriate expression of growth factors, kinases and possibly certain transcription factors. Our project focuses on the potential role of a family of transcription factors, the NF-kB/Rel family, in human breast cancer. Additionally the project will analyze a role for the so-called VNTR elements in the hereditary susceptibility of women to breast cancer and on a role for estrogen in modulating cell growth of human breast cancers. We have found that approximately 35% of human breast cancers overexpress an important member of the NF-kB family (the p65 or RelA factor). Present efforts are aimed at understanding a role for this overexpression and at determining the type of tumor that is associated with this overexpression. Additionally, we have described an interesting new function of mutant/oncogenic forms of the tumor suppressor p53. Our analysis of VNTR elements has shown that bind to the important transcription factor AP-1. Future experiments are aimed at understanding a functional role for this binding to a DNA element that is associated with human breast cancer. Additionally we have found that a constitutive form of NF-kB also binds these important elements.

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Albert S Baldwin 7/27/95

PI - Signature Date

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INTRODUCTION:

Human breast cancer is characterized by altered expression of growth factors, growth factor receptors and kinases (Lippman amd Dickson, 1989). Downstream modulators of growth factors and kinases are transcription factors which likely modulate altered responses in breast cancer. Genetic analysis also indicates that other factors are involved in development or progression of human cancer, including repetitive DNA elements called VNTR elements (Krontiris et al, 1993) the recently cloned BRCA1 gene (Futreal et al, 1994), and the tumor suppressor gene p53 (Rotter et al, 1993). Additional emphasis has been placed on the role of estrogen in the development of breast cancer.

NF-kB/Rel proteins are widely distributed and are typically found in the cytoplasm where they are associated with an inhibitor protein called IkB (Beg and Baldwin, 1993). Upon exposure to inflammatory cytokines or growth factors, NF-kB factors dissociate from the IkB inhibitory proteins and translocate into the nucleus (Finco and Baldwin, 1995). In the nucleus, these dimeric factors regulate transcription of genes that contain the kB binding site. Typically these genes encode proteins involved in immune and inflammation responses but more recent work has shown that genes encoding growth regulatory proteins (such as c-myc) are regulated by NF-kB.

NF-kB and IkB proteins are associated with oncogenesis. For example, members of the NF-kB family of proteins are related to the product of the c-Rel proto-oncogene which is found overexpressed in certain tumor cell lines. Additionally, the p100 member of the NF-kB family is found translocated in certain lymphomas as is Bcl-3, a member of the IkB family (see Beg and Baldwin, 1993). Based on our preliminary data, we have proposed that dysregulation of normal NF-kB regulation (i.e., chronic nuclear localization of some forms of NF-kB) may play an important role in the development or progression of human breast cancer.

We have previously found that a breast cancer cell line exhibited constitutive activation of NF-kB. Furthermore, we had found that estrogen treatment of a fibroblast caused enhanced expression of a reporter gene that is controlled by multiple NF-kB sites. Furthermore, some genes shown to be overexpressed in human breast cancer (for example, vimentin and ICAM-1) are known to be regulated by NF-kB. Thus one broad aim of the proposal (Dr. Baldwin's part of the project) was to study the expression of NF-kB/Rel proteins in human breast cancer and to study the functional outcome of this potential overexpression.

A second focus of this proposal is the functional study of human VNTR elements. These DNA elements are repetitive seqences and certain rare alleles of these repeats are associated with an increased risk in the development of breast cancer (Krontiris et al., 1993). These elements arise from the head to tail concatenation of short sequence motifs. The proto-oncogene Ha-ras1 is tightly linked to a VNTR. This VNTR consists of 30-100 copies of a 28 bp DNA element. Krontiris first showed that rare alleles of Ha-ras appear in the genome of cancer patients at a higher frequency that in non-affected women. More recent data indicate that these rare alleles are found more frequently in African-Americans and are correlated with an increase of breast cancer in this population. It has been published that VNTRs bind to NF-kB. The broad aims of this part of the proposal (Dr. Conway) are to explore a role for VNTR elements in human breast cancer, exploring the correlation of rare alleles with breast cancer patients and further characterizing the factors that bind to VNTR elements.

Aim 1 is to investigate the potential biological function of the Ha-ras VNTR through characterization the nuclear factors that bind to this element with a definite focus on the potential interaction of NF-kB/Rel proteins. Further approaches include studies aimed at addressing potential transcriptional activation properties of the VNTR.

Aim 2 is designed to determine if NF-kB/Rel binding to VNTR elements may be used as a more refined method of identifying patients at risk for breast cancer.

Aim 3 is to analyze relative nuclear and cytoplasmic levels of NF-kB/Rel proteins in normal breast epithelium and in human breast cancer. We will correlate NF-kB expression with activation of certain kinases thought to regulate NF-kB expression and with the status of transcriptional activators shown to regulate NF-kB gene expression.

Aim 4 is to correlate expression of NF-kB with expression of known or suspected prognostic markers for human breast cancer (ICAM-1, urokinase and vimentin). We will determine if the ligand for HER2/Neu, NDF, can induce the expression of NF-kB.

Aim 5 is to determine whether estrogen can regulate gene expression through a kB site and whether this is due to the activation of NF-kB/Rel binding activity.

BODY:

Conway Laboratory:

Progress/Results:

Specific Aim 1: Transcriptional Regulatory Function of the Ha-ras VNTR A direct role for high risk rare Ha-ras alleles in breast cancer would require that these alleles possess biological functions different from the low risk common alleles. Certain studies indicate that rare Ha-ras alleles bind NF-B transcription factors more readily, thereby effecting a higher level of transcriptional enhancement than the common alleles; this difference may be dependent upon their respective internal sequences. We envision that the G/C polymorphisms within the 28 bp repeat subunit could potentially affect NF-B protein binding, perhaps by influencing methylation patterns or DNA secondary structure (see MVR studies of specific aim 2).

If we are able to more clearly define the differences between rare and common alleles based upon a combination of transcription factor binding and structural characteristics such as VNTR length and internal sequence variations, we can identify the true rare alleles which predispose to breast cancer. In addition, we are most interested in identifying characteristics of the rare alleles in our black breast cancer cases, since they possessed the largest number of these alleles.

To this end, we proposed to investigate the potential regulatory role of the Ha-ras VNTR by characterizing its interaction with members of the NF-kB/rel family and other transcription factors. This was to be accomplished by evaluating protein binding to 28-bp VNTR subunits carrying specific G/C polymorphisms, and to longer tandem arrays of subunits generated by MVR from either common or rare alleles. We were also interested in characterizing the binding proteins present in a series of nuclear extracts from cell lines and from breast tumor tissues as well as normal mammary epithelium. Finally, we wanted to determine the biological outcome of VNTR/protein interactions by characterizing transcriptional regulatory activity of common versus rare VNTR sequences in CAT assays.

Protein Binding to Ha-ras VNTR Repeats We have begun to characterize the interaction of the Ha-ras VNTR with members of the NF-B/rel family of transcription factors. All prior VNTR binding utilized either a single oligomeric sequence or a mixture of the various 28 bp repeat types prepared from BstN1 restriction digests of a plasmid containing the VNTR. It was unclear whether one of the four repeat types in this mixture might be responsible for the majority of the observed binding. We have therefore synthesized double-stranded 28 bp oligonucleotides corresponding to each of the 4 common VNTR repeat types carrying specific polymorphisms. In order to replicate the binding experiments of Trepicchio and Krontiris (1992), we began with 28 bp fragments starting at position 19 (the BstN1 cleavage site) instead of position 1. The reason for this was that, according to Krontiris, the putative NF-kB site spanned the junction of adjacent repeats. These DNA fragments were end-labeled and used in mobility shift assays to determine which repeat type is bound most tightly by proteins in nuclear extracts. Conditions for these experiments were established using nuclear extracts from Jurkat T cell leukemia cells either untreated or following induction with a combination of phytohemagglutinin (PHA) and phorbol myristate acetate (PMA).

As shown in Figure 1, gel shifts using the BstN-4 repeat type 4 28 bp Ha-ras VNTR probe indicate that this sequence does indeed exhibit protein binding profiles that appear to correspond to NF-kB/rel proteins. Two bands of similar size are visible in all lanes except lane 1 which is probe alone without nuclear extract. Protein binding to the VNTR sequence was the same for both the induced and the uninduced extracts, indicating that only the constitutive form of NF-kB (p50) appears to bind this sequence. This experiment was repeated using the BstN-1 type 1 repeat probe with similar results (data not shown), suggesting that at least for these repeats, there is no differential binding of proteins.

In an attempt to demonstrate the specificity of binding to the Ha-ras VNTR sequence, we used the an AP-1 consensus sequence as unlabelled competitor. The transcriptional regulatory factors of the fos and jun families bind to AP-1 sites. As shown in Figure 1 lanes 4 and 5, the AP-1 sequence competed away protein binding primarily in the lower band but not of the upper band. Alternately, when the NF-kB consensus sequence, UV-21, was used as competitor, the upper band of protein binding disappeared (lanes 6 and 7). These results suggest that the Ha-ras VNTR contains an AP-1 as well as an NF-kB site. In addition, the fact that the UV-21 (NF-kB) and AP-1 competitors reduced binding in different bands suggest that the binding sites may be separated along the length of the repeat.

Because proteins that might normally bind AP-1 sites (such as fos and jun) may also bind the Ha-ras VNTR, we carried out additional gel shifts to evaluate the binding of proteins directly to AP-1. Figure 2 shows the binding patterns obtained using both an AP-1 (lanes 1-5) and a BstN-1 probe (lanes 6-10, similar to figure 1). Unlike the Ha-ras sequence, AP-1 binds both constitutively expressed (uninduced) proteins and proteins induced following treatment with PHA and PMA (lanes 2 and 3). The UV-21 sequence efficiently competes for binding in the lower constitutive protein band, but not in the upper induced band. The AP-1 and the UV-21 competitors both eliminated binding to the BstN-4 VNTR sequence (lanes 8 and 9).

Specific Aim 2: Improved methods of identifying high risk rare alleles by their variant structure In order to improve our ability to identify high risk Ha-ras alleles associated with breast cancer, we have devised a PCR allele length assay to increase allelic resolution, and have characterized internal sequence differences between low risk common alleles and high risk rare alleles using MVR. These approaches are being used together with any transcriptional regulatory differences identified in Specific Aim 1 to screen two breast cancer case-control populations for high risk alleles. The subjects of the Garrett study have already been allelotyped using these methods and the preliminary results are discussed below.

Length Typing of Ha-ras Alleles by PCR All previous studies examining the association between rare Ha-ras alleles and cancer utilized Southern hybridization techniques, which cannot adequately resolve alleles differing by one or a few repeats. We therefore developed a PCR-based technique to permit direct sizing of the Ha-ras VNTR. The Garrett study population which consists of a total of 607 breast cancer patients and controls (representing 1130 alleles), was originally Ha-ras allelotyped by Southern blotting. PCR re-typing of this data set uncovered a very significant degree of mistyping. Of 674 alleles judged to be a1 common alleles, 42 (6.2%) vary from the a1 length by 1 or 2 repeat units. The degree of mistyping was 17/130 (13.1%) for a2, 25/116 (21.6%) for a3, and was 66/119 (55.5%) for a4. We conclude that the PCR method is much more accurate than previous Southern blotting approaches and can distinguish VNTR alleles differing by as little as one repeat unit. Our statistical re-analysis of the Garrett data set is expected to be complete in the next two weeks and we will then compare breast cancer risk due to rare alleles identified by Southern blotting or PCR.

Minisatellite Variant Repeat (MVR) Analysis of VNTR Structure Minisatellite alleles vary not only in repeat copy number but also in the interspersion pattern of repeat sequence variants along the VNTR. Internal structure may provide clues to the genesis and/or function of the rare VNTR alleles and therefore may better correlate with cancer development. The Ha-ras VNTR sequence derived from the EJ bladder carcinoma cell line reveals either a G or C at positions 7 or 15 in the 28 bp repeat. VNTR sequence variants are readily detected by a modification of the minisatellite variant repeat (MVR) coding approach. Four repeat-specific primers corresponding to the G/C polymorphisms and a common anchored primer outside the VNTR are used to PCR amplify fragments whose lengths define the positions of the polymorphisms in the repeat unit. In effect, an allele-specific sequence polymorphism ladder is generated. Using a non-radioactive version of MVR for rapid screening of DNA samples, we are able to type 20-30 repeat units from the 5' end of the VNTR (Figure 3).

We have used MVR mapping to define the range of sequence allelotypes present in 385 breast cancer cases and medicine clinic controls of the Garrett population (Garrett et al, 1993). The results of these studies are summarized in Tables 1-4. The analyses were begun on a1, a2, a3 and a4 homozygotes since they display the simplest MVR patterns, having only a single band present at each ladder position. The first 6 repeats of all alleles analyzed thus far show exactly the same MVR pattern which is: 5'-0,0,1,3,2,1,..., with "0" representing null repeats that contain additional mutations preventing specific binding of primers. Only 2/385 individuals exhibited mutations (repeat insertions, deletions, etc.) within these first 6 repeats. Beginning at repeat 7, the MVR patterns of the four common alleles diverge such that each allele then displays its own unique "signature" sequence. Interestingly, the a1 and a2 alleles are composed of types 1, 2 and 3 repeats while a3 and a4 also have an additional type 4 repeat not found in a1 and a2. Unlike certain other VNTR loci, the Ha-ras VNTR did not demonstrate detectable alterations in repeat subunit length, nor did we commonly observe the introduction of multiple point mutations leading to the conversion of specific repeats to null or "0" type repeats.

Most of the subjects in the Garrett population were a1 heterozygotes (a1/a2, a1/a3, a1/a4, etc.). Once the a1 MVR pattern was firmly established, we deduced the pattern of the second common or rare allele by subtracting it from the dual pattern seen in these individuals. The MVR alleles represented in our population are depicted in Table 1.

Table 1: MVR Patterns of the Common and Intermediate Ha-ras VNTR Alleles in the Garrett Study

	A1.02 (A1-2)	A1a	A1b	A1.2 (A1+2)	A1.4 (A1+4)	A1.6 (A1+6)	A2	A3a	A3b	A3c	A3.5	A4
	0.95 kb⇓	1.00 kb		1.05 kb	1.10 kb	1.15 kb	1.45 kb		2.05 kb		2.45 kb	2.50 kb
	28→	30		32	34	36	46		69		85	87
	(1.8%)♥	(49.2%)	(7.0%)	(1.0%)	(1.3%)	(1.5%)	(10.1%)	(2.5%)	(2.3%)	(2.0%)		(11.1%)
5'	0	0	0	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0	0	0	0	0
	1	1	1	1	1	1	1	1	1	1	1	1
	3	3	3	3	3	3	3	3	3	3	3	3
	2	2	2	2	2	2	2	2	2	2	2	2
	1	1	1	1	1	1	1	1	1	1	1	1
	1	1	1	1	1	2 *	2	2	2	2	4	4
	1	3	3	3	3	2 *	2	1	1	1	1	1
	3	1	1	1	1	2 *	2	1	1	1	2	2
	1	1	1	1	1	1 *	2	3	3	3	2	2
	1	3	3	3	3	2 *	1	2	2	2	4	4
	3	2	2	2	2	1 *	3	1	1	1	1	1
	1	2	2	2	2	1	1	(4)	(1)	(4)	2	2
	3	1	1	1	1	3	1	1	1	1	3	3
	1	3	3	2 *	3	1	3	(2)	(2)	(3)	2	2
	2	1	1	2 *	2 *	1	1	2	4	2	4	4
	1	(1)	(2)	3	4 *	3	3	4	1	4	1	1
	1	(2)	(1)	1	1 *	2	1	1	2	1	2	2
	3	2	2	1	3 *	2	2	2	3	2	2	2
3'	1	2	2	2	1	1	3	3	1	3	2	2

A; common or , I; intermediate allele, ⇓; approximate allele length in kilobase pairs determined by Southern hybridization, →; number of repeat units determined by PCR, ♥; allelic frequency in the medicine clinic controls, ( ) subunit polymorphisms among the A1 or A3 subtypes, *; repeats added to the A1 length to generate the intermediate alleles, nd; not determined.

One important early finding which formed the basis for all subsequent studies is that for all alleles occurring repeatedly in the population (the common and intermediate alleles shown in Table 1), VNTR allele length is very tightly linked to MVR internal sequence. That is, nearly all alleles having the same length also have the same MVR allelotype. MVR also revealed abnormal sequences for several common length alleles. This technique therefore complements the length analyses and should enable us to more accurately detect variant alleles.

Additional MVR analyses are summarized in Tables 1-4. In Tables 1 and 3, the allele lengths shown were determined from Southern analyses in the original Garrett study. In Table 1, sizes are also given in numbers of repeat units determined by PCR for alleles between a1 an a2. The common alleles by length are designated A1, A2, A3 and A4. Several MVR subtypes exist for the a1 and a3 common alleles; these subtypes are indicated by letters. There are 2 subtypes of a1 allele, designated A1a and A1b, which make up 87.5% and 12.5% of all a1 alleles, respectively, and three subtypes of a3, called A3a, A3b and A3c, comprising 37%, 33% and 30% of a3 alleles, respectively. The A1b MVR allelotype is slightly more prevalent in the breast cancer cases, but this difference does not seem to be significant.

Table 2: Frequencies of Ha-ras MVR Allelotypes in Breast Cancer Cases and Controls

MVR Allele	CASES Number of Alleles (% of total)		MED. CLINIC CONTROLS Number of Alleles (% of total)	
Known patterns				
A1.02	3	(0.8)	7	(1.8)
A1a	172	(46.2)	196	(49.2)
A1b	36	(9.7)	28	(7.0)
A1.2	9	(2.4)	4	(1.0)
A1.4	3	(0.8)	5	(1.3)
A1.6	2	(0.5)	6	(1.5)
A2	35	(9.4)	40	(10.1)
A3a	16	(4.3)	10	(2.5)
A3b	11	(3.0)	9	(2.3)
A3c	5	(1.3)	8	(2.0)
A4	35	(9.3)	44	(11.1)
Aberrant patterns:				
Alleles having:				
A single change in repeat type	9	(2.4)	17	(4.3)
An addition of 1 repeat	10	(2.7)	3	(0.8)
A deletion of 1 repeat	7	(1.9)	5	(1.3)
Multiple repeat changes or inversions	9	(2.4)	10	(2.5)
Rare/unique MVR pattern	10	(2.7)	6	(1.5)
Total alleles	372		398	

We have also uncovered a series of intermediate frequency alleles, called A1.2, A1.4, A1.6, that are derived from a1 by the incremental gain of 2 repeat units (a1+2, +4 or +6 repeats). The positions of these additional repeats relative to the a1 pattern are identified in Table 1. Another intermediate allele, a1-2, called A1.02, appears to be related to either a1 or a2 because it contains only types 1, 2 and 3 repeats. The apparent stability of the intermediate allele sequences is consistent with the original finding that they did not confer an increased risk for breast cancer.

In contrast, alleles that were a1 $\pm$ 1 in length did not show a specific pattern of repeat unit insertion or deletion; instead, these variations appeared to be completely random suggesting they are in fact mutations, not stable alleles descended from a1. Other minor random VNTR mutations were also found, including changes in repeat type, inversions of two repeats and multiple aberrations within otherwise recognizable alleles (Table 2). The total number of alleles having any alteration from the exact patterns of the common or intermediate alleles was similar between cases (12% or 45/372) and controls (10% or 41/398).

MVR has also enabled us to discern the lineage of the a3.5 allele (2.45 kb in the Garrett study) found almost exclusively in blacks. Despite a frequency similar to a2 in black control populations, it was arbitrarily designated as an intermediate allele in previous studies (18,25). MVR indicates that a3.5 possesses the same internal structure as the a4 common allele (2.50 kb), and therefore probably originated by truncation of this allele by approximately 500 bp. The

emergence of a specific, stable MVR pattern for the a3.5 allele suggests it should be considered a common allele in black populations.

In contrast to the common and intermediate alleles that were detected multiple times in the population, "rare" alleles possessed unique and highly disordered sequences and were each represented only once in our population of 385 individuals, with the exception of R6 which occurred twice (Table 3). We found a highly significant association between MVR pattern and allele length ($p<0.00001$); 94% of alleles with rare MVR patterns had rare lengths (15/16 alleles)

Table 3: Rare Ha-ras VNTR Alleles with Unique MVR Patterns in the Garrett Study

	R1 (#37)	R2 (#227)	R3 (#529)	R4 (#166)	R5 (#136)	R6 (#29,415)	R7 (#421)	R8 (#397)	R9 (#287)	R10 (#45)	R11 (#39)	R12 (#343)	R13 (#415)	R14 (#462)	R15 (#348)	R16 (#379)
	X	N	C	C	C	C	C	X	X	X	X	X	C	C	C	C
	1.225⇓	1.250	1.700	1.750	1.150	1.175	2.900	2.225	1.650	1.750	1.925	1.175	1.650	1.150	2.225	1.750
	A1+12→	A1+12	A2	A2-A3	A1+7	A1+8	>A4	A4	A2	A2-A3	A3	A1+6	A2	A1+2	A3-A4	A2-A3
5'	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	1 ?	**2** I4	2 ?	**4** A4	**1** A1	**2** A2	**4** A4	**4** A4	2 ?	**4** A4	**2** A3	**1** A1	**4** A4	**4** A4	**2** A3	2 ?
	1	**2**	3	**1**	**3**	**2**	**1**	**1**	3	**1**	**1**	**3**	**1**	**1**	**1**	3
	1	**2**	1	**2**	**1**	**2**	**2**	**2**	1	**2**	**1**	**1**	**2**	**2**	**1**	1
	1	**1**	1	**2**	**1**	**2**	**2**	**2**	1	**2**	**3**	**1**	**2**	3	**3**	1
	3	**2**	3	**4**	**3**	**1**	**4**	2	3	**4**	**2**	**3**	**4**	1	**2**	3
	3	**1**	2	**1**	**2**	1	**1**	2	2	**1**	**1**	**2**	**1**	1	**1**	2
	1	**1**	1	**2**	**2**	3	**2**	2	1	1	**1**	**2**	**2**	3	2	1
	1	**3**	4	**3**	**1**	1	**3**	1	4	3	**1**	**1**	**3**	2	1	4
	3	**1**	2	1	**3**	2	1	3	3	1	**2**	2	1	2	1	2
	1	3	3	3	**1**	3	3	1	2	3	**4**	2	3	1	3	3
	1	1	3	3	**1**	1	3	1	4	3	2	3	3	3	2	2
	3	2	4	2	3	2	2	1	1	4	1	1	4	1	1	4
	1	3	1	4	2	1	4	2	2	1	3	3	4	1	2	1
3'	1	1	2	1	2	3	1	4	2	2	4	1	1	2	1	2

Allele sizes are given in: ⇓;kilobases, derived by Southern analyses, →;number of repeats from the A1 allele for R1, R2, R5, R6, R12, and R14 or predicted derivation of this allele based on size. For reference, the common allele sizes derived by Southern analyses were 1.00 kb for a1, 1.50 kb for a2, 2.00 kb for a3 and 2.50 kb for a4. Bolded repeats correspond to the common MVR allele to the right of each pattern, or are unrecognizable. Unbolded regions do not resemble motif of any known allele. C; case, X; medicine clinic control, N; breast referral clinic control.

Table 4: Rare Ha-ras Alleles By MVR and Length in Breast Cancer Cases and Controls

Rare Alleles Detected by MVR and/or Length	CASES Number of Alleles Combined (% of total)	CASES Black (% in populations)	CASES White (% in populations)	MED. CLINIC CONTROLS Number of Alleles Combined (% of total)	MED. CLINIC CONTROLS Black (% in populations)	MED. CLINIC CONTROLS White (% in populations)
♥Rare MVR/Rare Length	10	5	5	6	1	5
Normal MVR/Rare Length-Longer than MVR Predicts	7	2	5	3	0	3
⇓Normal MVR/Rare Length-Shorter than MVR Predicts	8	2	6	3	1	2
Number of Alleles Analyzed	372	46	326	398	48	350
Total Rare Alleles	25 (6.7)	9 (19.6)	16 (4.5)	12 (3.0)	2 (4.2)	10 (2.9)

♥Rare alleles that are apparently recombinant alleles begin at the 5' end as a known allele then convert to either a second recognizable pattern or a unique motif; Rare alleles that are presumed to be recombinant alleles because they have normal MVR patterns but their lengths are longer than their MVR allelotype would predict; ⇓Rare alleles by length only; not likely to be hybrids because lengths are shorter than MVR allelotype would predict, suggesting they were generated by truncation. Refer to text for odds ratios.

as determined by Southern analyses, while only 0.1% (1/754 alleles) of common length alleles harbored rare MVR patterns. Approximately half of the rare length alleles did not exhibit aberrant MVR patterns most likely because our limited characterization of 20 repeats could not detect mutations occurring in the center of the VNTR or farther downstream toward the 3' end. The a1 allele is composed of 30 repeats, while most rare alleles tend to be between a2 and a4 in length (46-86 repeats). Therefore, the 20 repeat MVR analysis would be more likely to uncover sequence abnormalities in the small alleles but would be biased against detecting rare alleles in the larger size range. A more rigorous MVR approach, ideally involving MVR determinations for the entire length of each allele, is required for the detection of all rare alleles.

Most rare alleles with unique MVR patterns began at the 5' end as one common allele, usually a4, then abruptly switched to either a second recognizable motif (R14 begins as a4, switches to a1) or became completely unrecognizable (R8, R14). This suggests that rare MVR alleles arise via recombination involving segments of one or more of the known alleles.
Certain rare alleles (R1, R3, R7, R16) bore no resemblance at the 5' end to any known allele, indicating that a complex series of mutations may have contributed to their formation.

The a4 allele appears to be the most unstable common allele, giving rise to the majority of the rare alleles with aberrant MVR patterns. Previous lineage studies by Krontiris indicated that all rare Ha-ras VNTR alleles maintained 5' flanking sequences in absolute linkage disequilibrium with the common allele nearest in size. Our MVR results, however, do not necessarily support this observation. To facilitate comparisons of MVR sequence with allele length, we have shown in Table 3 the rare allele sizes in kb derived by Southern blotting and also indicate either the nearest common allele or the two common alleles between which the rare allele lies. The R15 rare allele is closest to a1 in length, but begins at the 5' end as an a4 allele; according to Krontiris, it should possess an a1 motif instead of a4. Similarly, both R4 and R10 are between a2 and a3 in length, but their 5' motifs are from a4.

Using MVR criteria alone, we found an overall 2 fold excess of rare alleles in breast cancer patients as compared to controls. Because MVR and length analyses may better define the "affected" individuals, we asked if subjects with either rare MVR or rare length alleles are at risk for developing breast cancer. Our analysis shows that the combination of MVR and length analyses shows a statistically significant increase in risk for breast cancer (OR=2.3, 95% CI(1.15-4.68) (Table 4). In the white population a slightly increased risk was observed (OR=1.8, 95% CI(0.79,3.93), while the black breast cancer cases exhibited a disproportionate number of rare alleles, OR= 5.6, 95% CI(1.14,27.49), consistent with the original Garrett study findings based solely on VNTR length. Rare alleles fell into one of three classes: (1) alleles having both rare length and rare/unique MVR patterns suggestive of recombination (2) alleles with common or intermediate MVR patterns but with rare lengths that are longer than the MVR patterns would predict, indicating they were also probably derived by recombination, e.g., an a1 MVR but a near-a4 length, (3) alleles with common MVR but rare lengths that are shorter than the MVR predicts, suggesting that these were likely derived by truncation of larger alleles, e.g., an a4 MVR but a near-a2 length.

The finding that rare Ha-ras alleles with unique MVR patterns may have arisen by rearrangements of internal sequences is consistent with the observations of Alec Jeffreys for other VNTR. In addition, all three of the VNTR loci characterized by Jeffreys et al (1994) have exhibited polar mutations, usually in the 3' end. The fact that only 50% of rare alleles with abnormal lengths have sequence abnormalities within the first 20 repeats at the 5' end, suggests that the opposite end may harbor substantial variability. In support of this, most of the a1$\pm$ 1 alleles (29-31 repeats) in our study failed to show the additional or deleted repeat within the first 25 repeats at the 5' end, indicating that the mutations probably occurred close to the 3' end. It is therefore likely that if the Ha-ras VNTR undergoes mutations in a polar fashion like other VNTR loci, then the 3' end could be the hypermutable one. We are already expanding our MVR analyses to type close to 60 repeats using hybridization methododolgies at the 5' end and also adapt these techniques to evaluate sequences at the 3' end. Given that the largest VNTR allele is about 100 repeats in length, we should be able to completely characterize the entire length of all VNTR alleles which would greatly facilitate our detection of rare or recombinant alleles.

CONCLUSIONS:

1. Rare Ha-ras Alleles Distinguished by Variant Internal Structure The internal architecture of the Ha-ras VNTR provides clues to the derivation of these alleles. MVR studies in the Garrett population demonstrated that about half of the rare length alleles possess abnormal internal sequences that are indicative of recombination. Such recombination may lead to altered function in the rare alleles. We suspect that all rare length alleles actually have disordered MVR patterns, but the limited analyses of 20 repeats at the 5' end of the VNTR is not sufficient to reveal mutations occurring farther downstream or at the 3' end. Therefore, development of 3'-MVR methodology should enhance our detection of rare variants.

The PCR allele length and MVR assays have permitted us to more accurately identify common and rare length alleles. Our re-analysis of the Garrett data set using these methods is nearly complete. We will then move to the CBCS population which is recruiting 800 breast cancer cases, half of which are African American. Because the risk of breast cancer associated with a rare allele is far higher in blacks than in whites, we will be concentrating on analyses of the African American subjects in this population. In order to determine possible links between the germline presence of rare Ha-ras alleles (by length and/or MVR) and other known or suspected risk factors for breast cancer, the following information will be available from interviews of subjects in the CBCS: family history of cancer, complete reproductive history, hormone use, alcohol and tobacco use, occupational exposures, pesticide exposures, and socio-demographic characteristics. In addition, a broad range of molecular analyses will be performed both on germline and tumor DNAs. Therefore, Ha-ras allele status can be correlated with a large number of molecular, epidemiologic and tumor characteristics.

2. The Ha-ras VNTR binds transcription factors Our gel shift experiments suggest that AP-1 binding proteins, in addition to certain NF-kB subtypes, bind to the Ha-ras VNTR. These results have important implications for the function of the Ha-ras VNTR and its role in tumor development, since the AP-1 binding proteins will likely belong to the fos and jun families of transcription factors. Because of these encouraging preliminary findings, our future studies will certainly focus on the possible interaction of both AP-1 binding proteins and NF-kB with the VNTR. However, we must first confirm our results by preforming a series of supershift experiments to unequivocally identify the protein species which bind the VNTR. We possess a panel of antibodies specific for the entire family of known NF-B proteins (p50, p52, p65, p49 and c-rel) and several AP-1 binding proteins (c-fos, c-jun and fra-1). We will also repeat the gel shift assays using a series of non-specific competitor DNAs to demonstrate the specificity of protein binding for either the Ha-ras VNTR or AP-1 sequences.

The interaction of different classes of transcription factors appears to be a rather unique and only recently reported phenomenon. In 1993, Stein et al (1993) described the 'cross-coupling' or direct physical interaction of the p65 subunit of NF-kB with c-fos or c-jun. This complex exhibited enhanced DNA binding and biological function via both the kB and AP-1 response elements. More recently, Giuliani et al (1995) described a complex consisting of the p50 subunit of NF-kB and fra-2 (a fos family protein) which binds to enhancer A of the major histocompatibility class I gene complex. The supershift experiments described above should enable us to dissect out the various components of the VNTR binding complex. In addition, we will compare the binding of transcription factors in nuclear extracts from the more biologically relevant cell types, breast tumor cell lines and primary human mammary epithelial cells.

We expect that the pattern of repeats comprising the VNTRs will be important both to the binding and the function of the region. We have found that the common VNTRs each possess a unique pattern of repeats. The deviant repeat patterns found in the rare variants might result in aberrant functioning of the region. The MVR technique enables us to synthesize a series of tandem repeat units directly from DNA samples containing common or rare alleles. These long MVR arrays more closely approximate the DNA binding sequences available in the intact

VNTR. By using these in binding experiments, additional proteins which require DNA secondary structure may be detected.

Finally, we will evaluate the outcome of the minisatellite/protein interactions by comparing the transcriptional regulation by the common and rare VNTR sequences in a series of CAT assays. We will determine which forms of NF-kB mediate transcription through the Ha-ras VNTR. We have already TA-cloned a series of rare alleles and these can easily be re-cloned into CAT vectors containing Ha-ras, IGF-2 (another chromosome 11p15.5 gene), RSV and c-fos promoters. These VNTR-containing reporters will be transfected alone or co-transfected with plasmids expressing the p50, p65, or rel proteins into cell lines that exhibit low basal levels of NF-B. CAT constructs will also be transfected into breast tumor cell lines BT-20 and T47D which express high nuclear levels of NF-B proteins.

The Ha-ras gene itself is the most likely target for transcriptional regulation by the VNTR and is certainly important in cellular growth and differentiation. In fact, the tyrosine kinase receptor Her-2/neu, which is amplified and overexpressed in many breast tumors, transduces its cellular signal via ras pathways. Several groups (Ohuchi et al, 1986) have reported overexpression of ras p21 proteins in human breast tumors, although the exact ras subtype was not identified. Antisera is now available from Oncogene Science which is specific for c-Ha-ras. We will therefore compare Ha-ras protein levels by immunohistochemistry in tumors derived from women with common or rare alleles.

Recently, a VNTR situated 5' to the insulin gene on chromosome 11p15.5 was found to control susceptibility to insulin-dependent diabetes mellitus (IDDM) type 1 at the IDDM2 locus (Kennedy et al, 1995; Bennett et al, 1995). In fact, this VNTR contains numerous high-affinity binding sites for the transcription factor Pur-1 and appears to regulate insulin transcription. IDDM is strongly associated with short VNTR alleles which possess less transcriptional activity than the longer VNTR alleles. Interestingly, this locus, like surrounding 11p15.5 loci, may be subject to genomic imprinting. These studies provide further evidence that other VNTRs in addition to the Ha-ras VNTR, possess biological activity and may be involved in human disease susceptibility.

BODY:

Baldwin Laboratory:

Progress/Results:.

Immunohistochemical analysis of NF-kB p65 expression in human breast cancer. We have made significant progress towards accomplishing the goals of Aim 3; that is, analyzing the expression of NF-kB/Rel proteins by immunohistochemical studies of human breast cancer sections. Utilizing the antibody against the human NF-kB p65 subunit, it was found that approximately 30-35% of human breast tumors express extraordinarily high levels of NF-kB, as compared to normal breast epithelium. Interestingly, the majority of these exhibit cytoplasmic levels of p65, although NF-kB is nuclear in several examples.

Table 5. Immunohistochemical Analysis of NF-kB p65 in Breast Cancer.

tissue type	negative	positive		total
		low	high	
normal breast	5	8	0	13
breast tumors	4	13	8	25

Figure 4 (appendix) shows an example of immunohistochemical staining for NF-kB p65 in human breast cancer. Figure 4A shows that normal human breast epithelium does not stain detectably with the antibody. Figure 4B shows one tumor with high levels of cytoplasmic NF-kB p65. Figure 4C shows another tumor that exhibits nuclear staining for NF-kB p65. Note that the cells were cross-stained (the purple staining) while the NF-kB staining is brown.

A second important aspect of this work is to understand why a relatively large number of tumors exhibit high levels of NF-kB p65. Preliminary data indicate that there is not a corresponding increase in cytoplasmic levels of the p50 subunit. Several models may account for the increase in NF-kB p65: loss of turnover of p65 or extremely high levels of expression of p65 mRNA. Further analysis will be to carefully determine if other forms of NF-kB/Rel proteins (such as p53, c-Rel or RelB) are overexpressed in breast cancer tissue.

We have recently begun to analyze the human p65 promoter and found that it contains potential binding sites for the tumor suppressor protein p53. Interestingly, approximately 40% of human breast cancer carries p53 mutations. We have recently shown that certain p53-like sites can regulate transcription in response to mutant forms of p53. Thus, it is possible that mutant forms of p53 activate the transcription of the p65 gene leading to high gene expression and accumulation of p65 in the cytoplasm.

A role for mutant p53 in regulation of gene expression. Progress has made in a new direction that is likely to be quite relevant to the issues described above, that is to understand a potential role for mutant forms of the tumor suppressor p53. p53 is an important growth suppressor and mutation of this protein is a major contributor to cancer (Rotter et al., 1993). Approximately 50% of all human tumors exhibit an alteration in p53. Typically this involves the loss of one allele and a missense mutation in the other (Olson and Levine, 1994). This evidence, along with data showing a function for mutant forms of p53, suggests that the missense mutations represent a gain of function phenotype. The mechanism whereby the mutant p53

functions is a mystery since it has been reported that mutant forms of p53 cannot bind to a p53 consensus DNA target site. We have found that mutant forms of p53 can activate gene expression through a site that resembles a p53 consensus site (Figure 5; Gualberto and Baldwin, 1995) Interestingly, this p53-like binding site is next to an Sp1 binding site and co-expression of mutant p53 and Sp1 resulted in enhanced gene expression and enhanced DNA binding of p53. Furthermore, it was shown that p53 and Sp1 can physically interact. Thus, it is proposed that mutant p53, which normally should not bind to DNA, may do so in the presence of Sp1 protein and a neighboring Sp1 binding site. These studies have relevance in potentially understanding the mechanism of elevated NF-kB p65 in human breast cancers and in understanding a role for mutant forms of p53 in cancer.

Efforts towards characterizing a functional role for BRCA1. We have begun to characterize the BRCA1 protein regarding a functional role. Dr. MaryLou McMaster has obtained BRCA1 protein encompassing the N-terminal region which is homologous to the so-called RING factor domain. In some proteins, this region has been shown to involve DNA binding. MaryLou has begun assays to determine if BRCA1 can bind to DNA and, if so, what the target specificity is. These experiments are just beginning and no definitive results have been obtained.

Goals for the upcoming year: (1) We will refine our analysis of NF-kB expression in tumors by determining if other NF-kB forms, such as p52, are overexpressed in human breast cancer and whether the inhibitor IkBa is overexpressed. (2) We want to analyze the differentiation and invasive status of the tumors to potentially correlate the NF-kB p65 overexpression with tumor phenotype. (3) In order to explain the overexpression of p65 in human breast cancers, we want to understand whether mutant forms of p53 can activate transcription of the p65 gene and whether the overexpression that we have detected is correlated with mutant forms of p53. This experimentation will involve caterogizing the NF-kB positive breast cancer tumors with mutations in p53. We will also explore a role for the mutant forms of p53 in the activation of NF-kB p65 by utilizing transfection and p65 promoter-reporter experiments.

(4) In the next year, we will begin to explore a role for signalling through a kB site relative to exposure of breast cancer cell lines to estrogen (this is Aim 4). Since we have shown that activated, oncogenic forms of Ras and Raf can activate gene expression through a kB site without nuclear translocation of NF-kB. We will determine if estrogen activation of a kB site is mediated by the Ras/Raf pathway. This will be tested by utilizing dominant negative forms of Ras/Raf and Map kinase. Additionally, we will determine whether estrogen can stimulate nuclear translocation of NF-kB and whether this is activated through the loss of the inhibitor protein IkBa.

(5) We will continue to try to determine if BRCA1 can bind to DNA and, if so, what the sequence specificity may be. These experiments utilize a randomized oligonucleotide approach that uses PCR to amplify bound DNA target sequences. Longer term goals would determine if BRCA1 is a transcriptional activator and, if so, what target genes may be regulated by this protein.

CONCLUSIONS:

1. The NF-kB p65 subunit is expressed at high levels in approximately 35% of human breast cancers. This is a significant figure and the underlying basis for this overexpression needs to be understood. It is interesting that the majority of these tumors exhibit cytoplasmic but not nuclear staining for NF-kB p65. It is possible that there is no role for overexpression of NF-kB p65 in the tumors that exhibit cytoplasmic levels. However, this overexpression may be indicative of an important event that would need to further characterized, for example the activation of a transcription factor that may up-regulate p65 transcription (such as mutant p53, see below). Elevated nuclear levels of NF-kB in certain human breast cancer (although relatively rare) may

indicate the activation of a kinase that is operative in inducing nuclear translocation of NF-kB. These data suggest a phenotypic difference in certain tumors relative to mechanisms that activate NF-kB.

2. Mutant (oncogenic) forms of p53 can activate transcription through certain sites that resemble p53 consensus elements and in association with the transcription factor Sp1. The importance of mutant forms of p53 in human cancers is not fully understood. Our data indicate that mutant p53 can activate transcription through certain DNA elements. This may be important in controlling the activation of p65 described above. It is also likely to be important in activating certain genes, for example that encoding basis fibroblast growth factor, that may be involved in the development of human breast cancer.

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Personnel Receiving Pay:

Albert Baldwin, P.I.

Kathleen Conway, Co-P.I.

Sharon Edmiston, Research Analyst

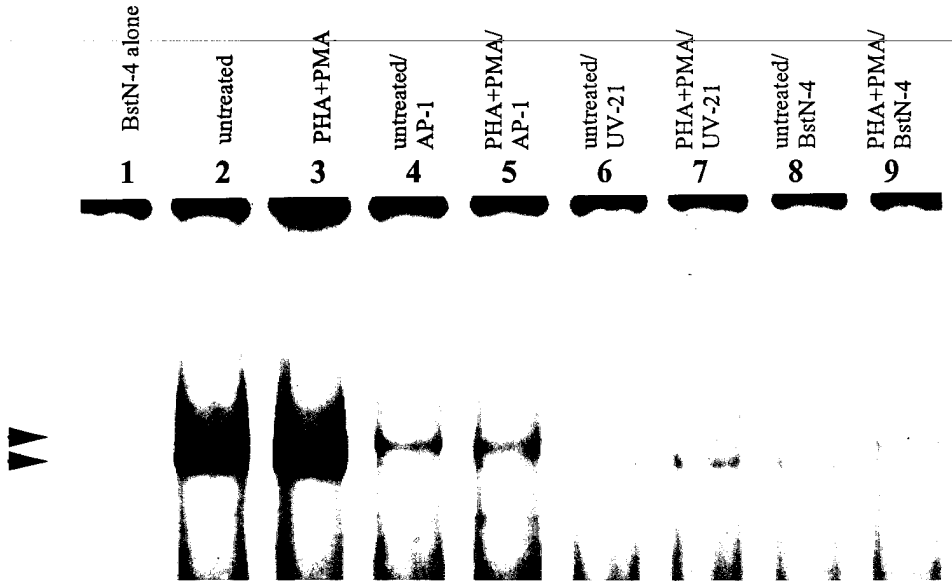
Mary Lou McMaster, Postdoctoral Fellow

Graduate Degrees Awarded

None

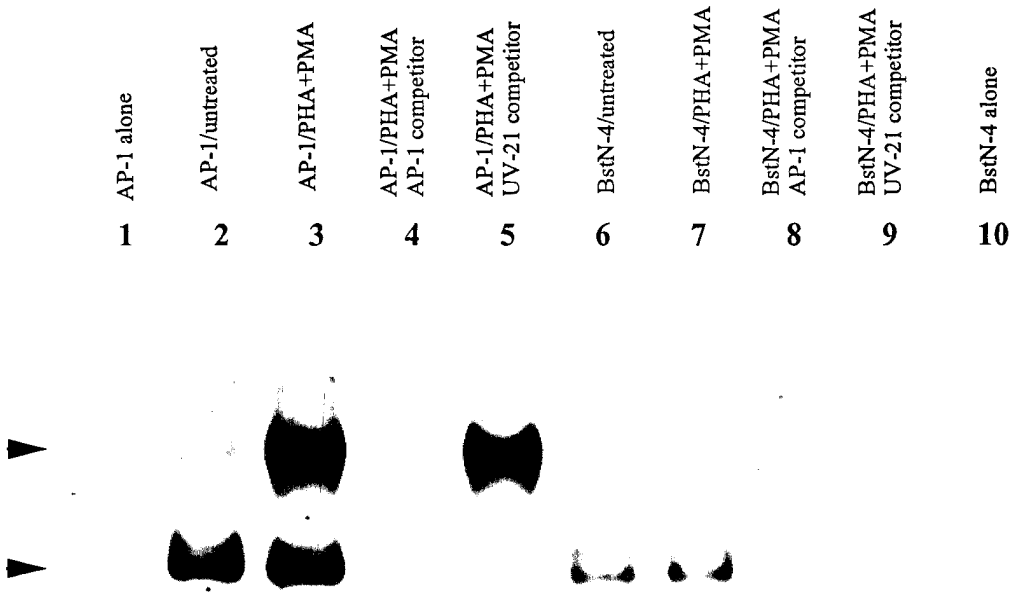
APPENDIX

Figure 1: Protein Binding to the Ha-ras VNTR Sequence



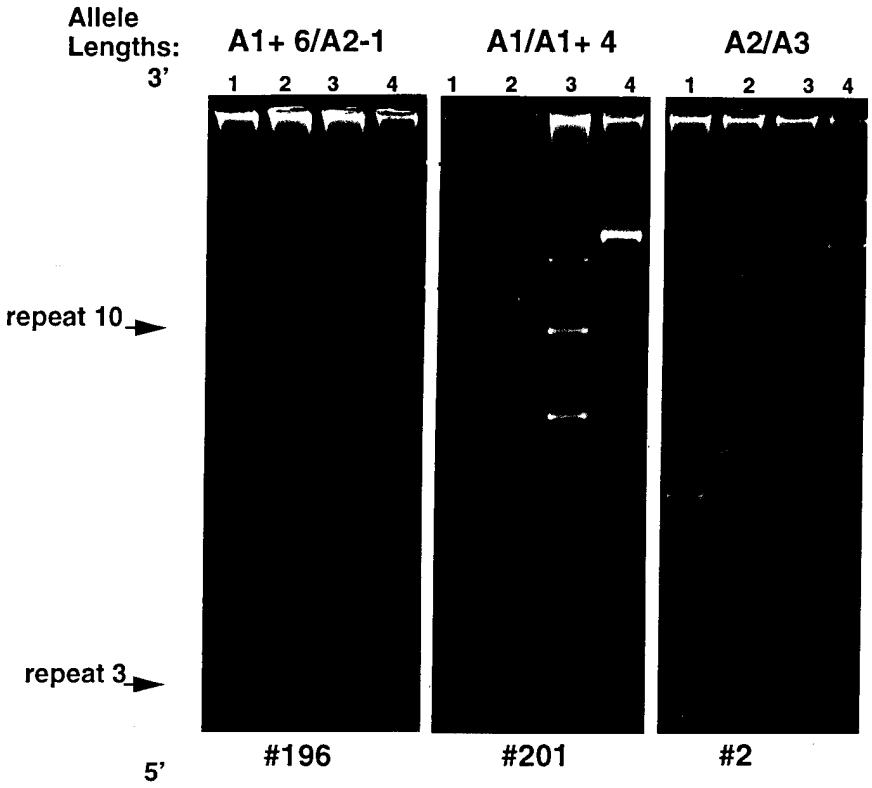
Gel shift assays were carried out using the BstN-4 labelled probe, derived from a Ha-ras VNTR repeat type 4, and crude nuclear extracts prepared from Jurkat cells either untreated or treated with PHA and PMA. Unlabelled AP-1 competitor was used in lanes 4 and 5, unlabelled UV-21 (an NF-kB concensus sequence) was added to lanes 6 and 7, and unlabelled BstN-4 was used in lanes 8 and 9. Arrows indicate protein binding to the Ha-ras VNTR sequence.

Figure 2: Protein Binding to AP-1 and Ha-ras VNTR Sequences



Gel shifts were conducted as described in Figure 2. Lanes 1-5 demonstrate binding to an AP-1 sequence, while lanes 6-10 show binding to the BstN-4 Ha-ras VNTR sequence. Unlabelled competitors are AP-1 or UV-21 (NFkB) sequences. Lower arrow indicates constitutive binding while upper arrow indicates binding of proteins to the AP-1 sequence from cells induced with PHA and PMA.

Figure 3: MVR Typing of Ha-ras VNTR Alleles in Heterozygous Subjects of the Garrett Study



Since the 5' end of each allele has the same 6 repeats, the MVR pattern begins as a homozygous pattern, then converts to a heterozygous pattern (showing two bands at these ladder positions).

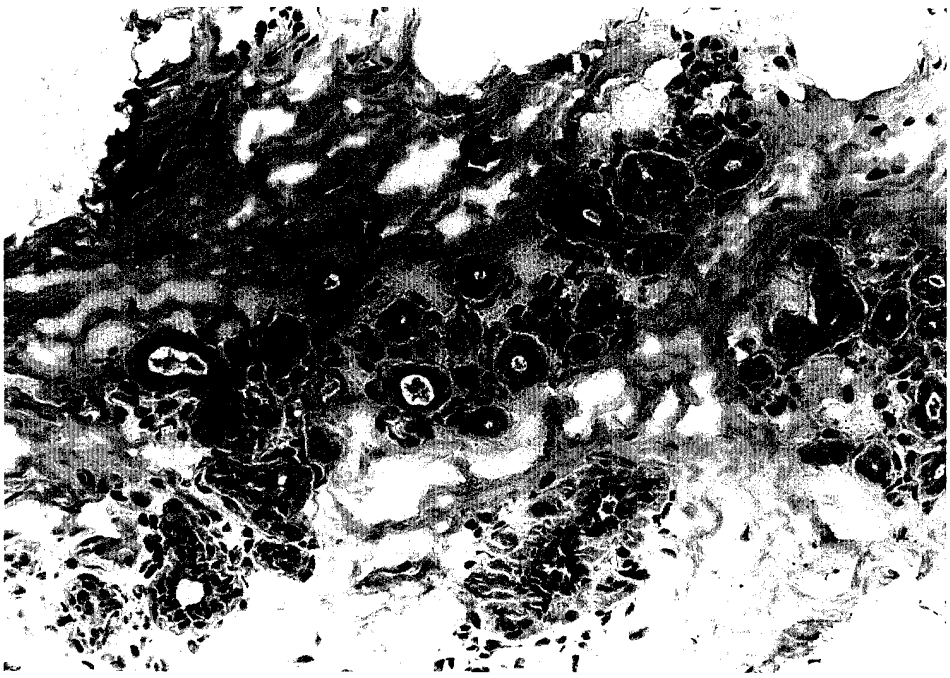


Figure 4A. Immunohistochemial analysis showing a lack of NF-kB p65 in normal breast epithelium. Purple staining is from the cross-stain and is not indicative of NF-kB.

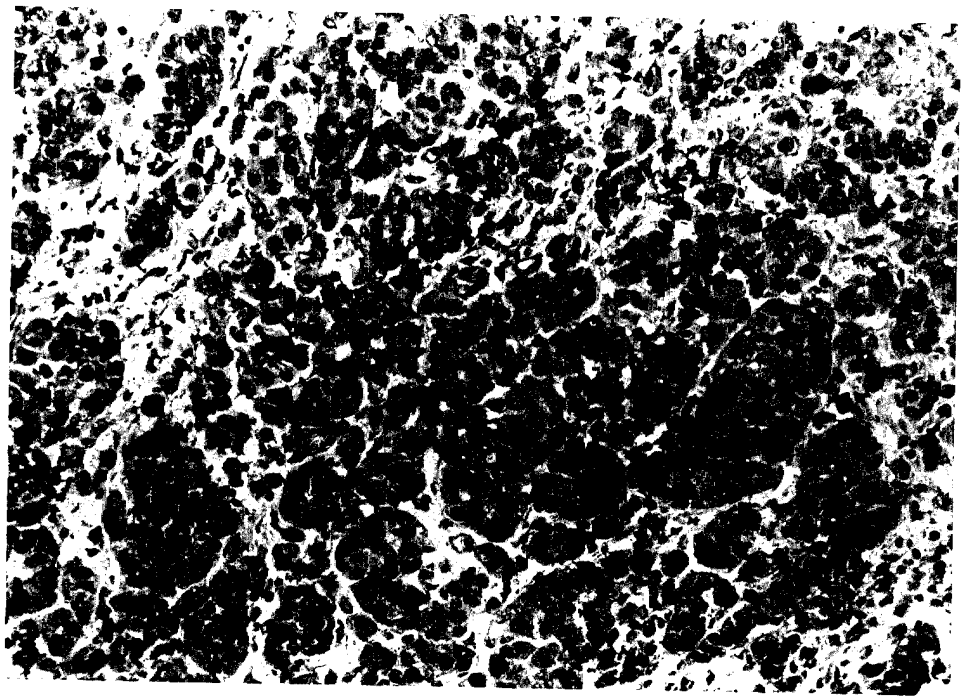


Figure 4B. Immunohistochemical analysis of NF-kB p65 in one breast cancer. Brown staining is specific for NF-kB p65 and, in this case, is largely cytoplasmic.

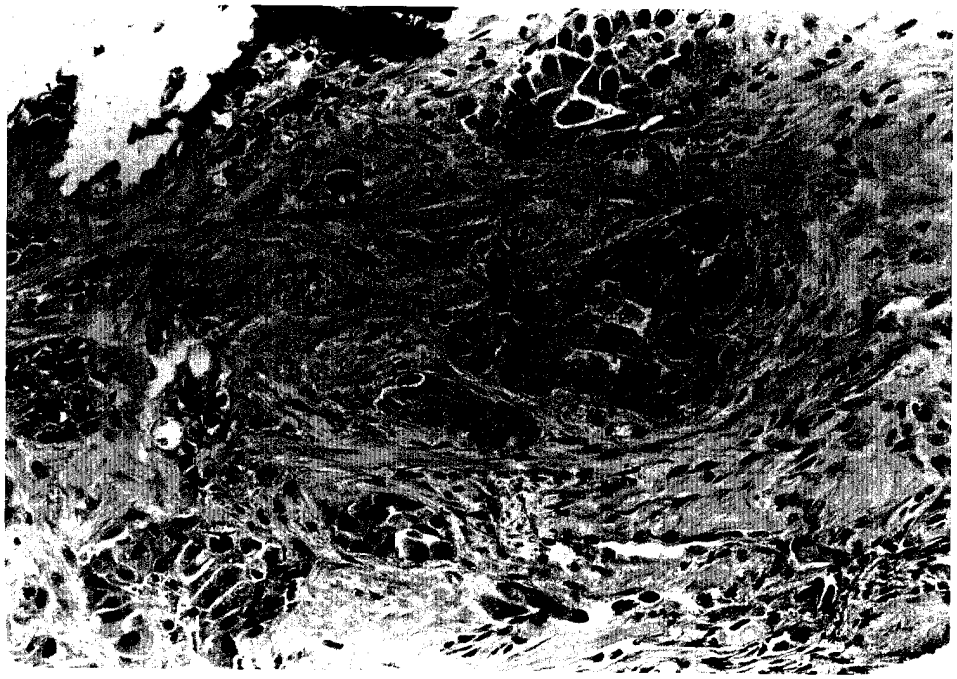


Figure 4C. Immunohistochemical analysis of NF-kB p65 in another breast cancer. Brown staining is specific for NF-kB p65 and is largely nuclear.

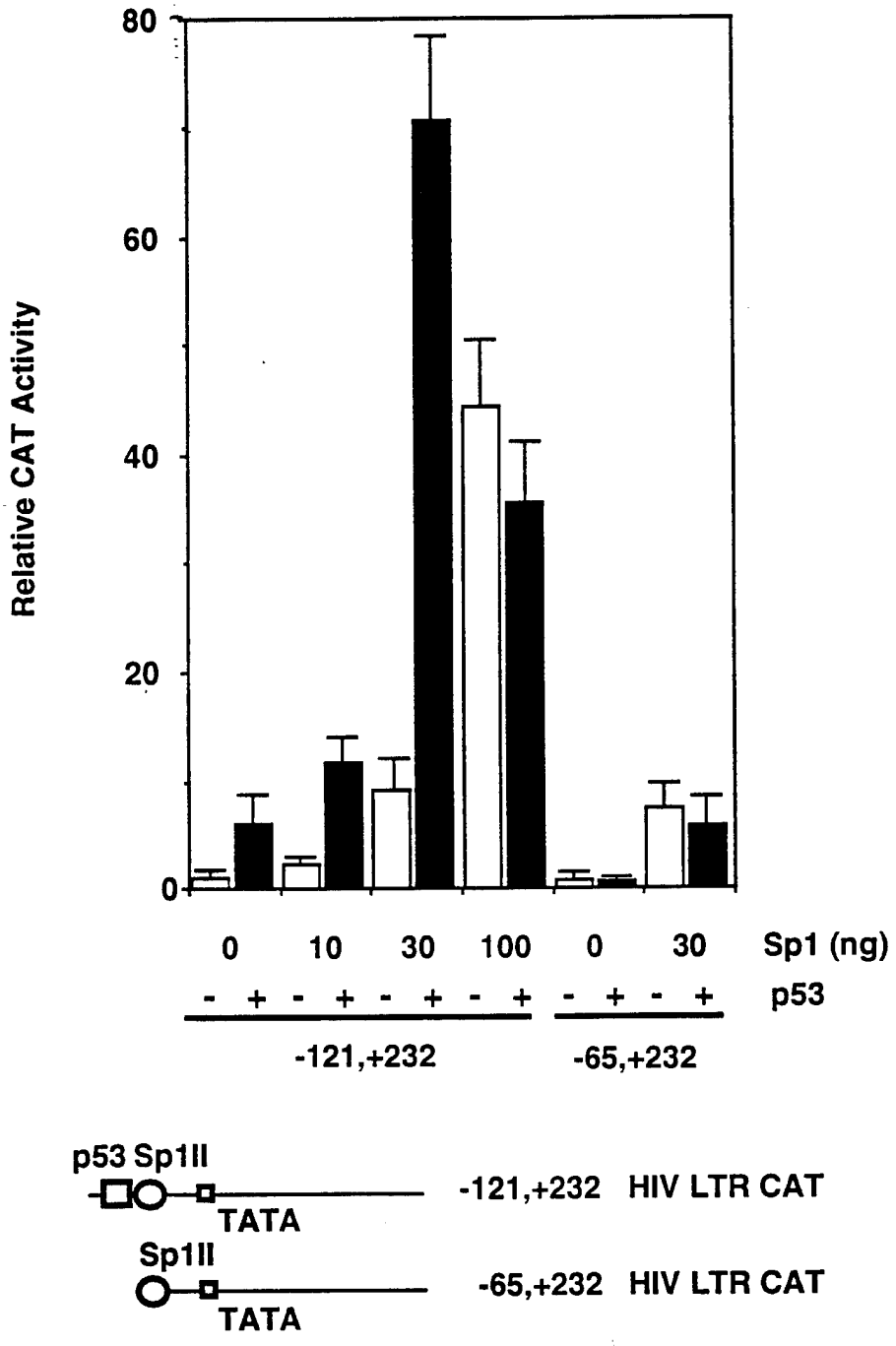


Figure 5. Activation of gene expression by mutant p53 and synergy with Sp1. Transfection of Sp1 and mutant p53 as indicated with either a construct containing the mutant p53 site (-121, +232) or one with this site deleted (-65,+232). Data indicate that p53 can activate gene expression on its own, but activation is much stronger with the co-expression of Sp1.